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(iv) contacting the lysed cells with various antibodies directed against the class I HLA antigens so as to form HLA-G isoform/antibody complexes, and

(v) establishing the HLA-G expression profile of said sample by detecting the complexes formed in step (iv).

REMARKS

Claims 2, 3 and 16-23 are active in the present application. Claim 3 has been amended for clarity. No new matter is believed to have been added by these amendments.

INFORMATION DISCLOSURE STATEMENT

Applicants again request that the Examiner consider that the document cited in the International Search Report, the request initially made on August 21, 2000 and again on October 22, 2001. A PTO Form 1449 was provided in the October 22, 2001 filing for convenience. Therefore, a returned, signed copy of the PTO Form 1449 is requested.

THE REJECTION UNDER 35 U.S.C.§ 102(b)

The rejection of Claims 2-3 and 14-23 under 35 U.S.C. § 102(b) over Bensussan et al is untenable for the following reasons.

The present claims provide methods of establishing the HLA-G expression of a **solid tumour** comprising several steps, the last of which is establishing the HLA-G expression profile of **the tumour sample itself**. Bensussan et al is concerned with detecting HLA-G expression in **cultured cells**.

In particular, on page 10292, column 2 to page 10293, column 1 Bensussan et al uses J25 cells, which are murine cells transformed with a human β_2m gene; J26-B7 cells, which are J26 cells transfected with HLA-B7 heavy chain gene; JEG-3, which express HLA-G; and TC9 cells, which are interleukin 2-dependent cloned tumor-infiltrating lymphocytes.

However, nowhere in Bensussan et al is there a description for establishing the HLA-G expression profile from a solid tumour as claimed.

As known in the art, selecting certain cells in culture, i.e., selecting a specific sub-population of cells that grow under culturing conditions, skews the cell types such that the cells are not likely to be representative of the solid tumor itself. Likewise, cell culture conditions typically change both the cell phenotype and gene expression profiles of the cultured cells, such that those cultured cells would not be representative of the actual HLA-G expression profile of the tumour. As evidence of the knowledge in the art, Applicants attach two publications. In Rueben[1]: "normal cells are relatively uniform when organized in a tissue, but become heterogeneous for many characteristics when they are dispersed and grown in monolayer culture...although phenotypic differences among cells of a given type may be transient, they can be perpetuated by protracted exposure to selective conditions." (See paragraph 2 of the Abstract). Likewise, Wolffe and Tata[2] describes that the "[i]solation of specialized cell types for the analysis of tissue-specific gene function often results in loss of the differentiated phenotype." (See the Abstract).

In sum, it is clear that Bensussan et al do not disclose the present methods of assessing the HLA-G expression profile of a solid tumour because as noted, *supra*, Bensussan et al discloses detecting HLA-G expression in specific cultured cells.

Withdrawal of this ground of rejection is requested.

THE REJECTION UNDER 35 U.S.C. § 112, SECOND PARAGRAPH

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[1]Rubin (1990) Cancer and Metastasis Reviews 9:1-20.

[2]Wolffe and Tata (1984) FEBS Letters 176(1):8-15.

The rejection of Claims 3 and 19-23 under 35 U.S.C. § 112, second paragraph is obviated by amendment.

Applicants submit that the present application is now ready for allowance. Early notification of such allowance is kindly requested.

Respectfully submitted,

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IN THE CLAIMS

Claims 1, 14, and 15. (Canceled).

Please amend Claim 3 as follows:

3. (Twice Amended) A method for establishing the HLA-G expression profile of a solid tumour with a view to selecting a treatment which is suited to said tumour or with a view to monitoring the evolution of said tumour, comprising:

(i) removing a tumour sample,

(ii) optionally, labeling the cells of said sample,

(iii) lysing the cells,

(iv) contacting the lysed cells with various antibodies directed against the class I HLA antigens so as to [possibly] form HLA-G isoform/antibody complexes, and

(v) establishing the HLA-G expression profile of said sample by detecting the complexes formed in step (iv).--

Cancer and Metastasis Reviews 9: 1–20, 1990.

The significance of biological heterogeneity

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There are good reasons to suspect that heterogeneity (i.e., variability within any given set of samples) is an essential *characteristic of organic life. This differs widely from the traditional view that heterogeneity is only a nuisance that is to be circumvented or otherwise eliminated. Hence, controversies and mutual misunderstandings of two groups adhering to these diametrically opposed viewpoints are just about inevitable.*

W.M. Elsasser
Proc. Natl. Acad. Sci. USA **81**, 5126-5129, 1984.
Outline of a Theory of Cellular Heterogeneity

Key words: phenotypic variation, progressive state selection, enduring modifications

Abstract

Heterogeneity of expression for a variety of characteristics is found among malignant cells in the organism and in culture. Normal cells are relatively uniform when organized in a tissue, but become heterogeneous for many characteristics when they are dispersed and grown in monolayer culture. The heterogenizing effect of growth in culture indicates that the morphology and behavior of normal cells is ordered by their topological relations in tissues and other homeostatic influences of the organism. Weakening of these ordering relations may contribute to malignant transformation, as it usually does in rodent cell culture.

Although phenotypic differences among cells of a given type may be transient, they can be perpetuated by protracted exposure to selective conditions. Examples are cited of selection which leads to an adapted state that is heritable for many cell generations after removal of the selective conditions. Such heritable adaptations are analogous to the Dauermodifikationen, or lingering changes, first described in ciliated protozoa and shown there to be under cytoplasmic control. The concept of *progressive state selection* is introduced to account for heritable adaptation at the cellular level. It depends on the spontaneous occurrence of transient, variant states and their successive selection to progressively higher levels of adaptation to an altered microenvironment. Although the process is basically epigenetic, it may be stabilized by genetic change. The concept is consistent with our present knowledge of tumor development, including progression to metastasis, and with epigenetic aspects of normal development.

Introduction

The theoretical physicist Walter Elsasser focused his attention for many years on the fundamental characteristics that distinguish living from non-living things. His analysis led him to the conclusion that the primary distinction lies in the immense heterogeneity of the molecular constituents of living things, down to the cellular level. By contrast, 'atoms, ions, radicals or (small) molecules of the same kind have the same physical characteristics (masses, charges, magnetic moments, spectral lines and so on) for measurements going to as many decimal places as one wishes to reach' [1]. A cell, since it is composed of an enormous number of intermingled or different molecular constituents, is a chemically heterogeneous system. While each reaction within a cell is theoretically resolvable to a

homogeneous chemical mechanism due to the involvement of only a few molecular species, it is impossible to extrapolate from this to 'explain' the behavior of the whole cell. At the level of the cell, many mechanisms operate simultaneously, and there is a mixing of homogeneous and heterogeneous aspects, which cannot be separated from each other operationally. From these beginnings, Elsasser develops the concept of *indeterminacy* which, for our present purposes, signifies that the cell can arrive at a succession of states, or any given state, by a variety of molecular paths, none of which is uniquely determined as causal for the biological state.

Elsasser is obviously concerned with molecular heterogeneity, but he recognizes that as the level of organization proceeds upward from the molecular level to the cell, tissue, and organism, there is increasing order. In the language of Paul Weiss [2], macrodeterminism dominates microdeterminism. Weiss had in mind well-characterized situations in embryological development in which the eventual location of large blocks of cells can be predicted accurately, but the fate of individual cells cannot. Thus, unpredictability exists at the cellular but not the tissue level, fostering the idea that hierarchical controls are operative in living systems. The fact that the dispersal of embryonic cells for monolayer culture results in a loss of their capacity for differentiation [3] is one clear example of the consequences of disrupting such hierarchical systems in organisms.

The term *ordered heterogeneity* was introduced to indicate the increase in order going up the scale of size in biological systems [1]. A striking example of the ordering tendency of tissue organization is seen by measuring the distribution of cell sizes as a function of time after chick embryo fibroblasts are dispersed for monolayer culture [4]. There is a tight distribution of cell sizes when measured 2 hours after seeding and attachment to the dish. Within 24 hours in growth medium, the size of the average attached cell increases more than four-fold, and the distribution of sizes has become very broad. The cells maintain their increased size and heterogeneity indefinitely during their sojourn in culture. Other expressions of heterog neity among normal cells in culture have since been demonstrated. They include the number of divisions attained by clones in culture before they senesce [5, 6], the intergenerational time of cells within clonal lines [7], and the enzyme content of clones and their subclones [8]. These investigations indicate that the release of cells from their three-dimensional arrangement in the animal allows them to express in their morphology, composition, and behavior the molecular heterogeneity postulated as a first principle of biology by Elsasser, and demonstrates the ordering effect of higher levels of organization.

Although cell culture was needed to demonstrate the potential for heterogeneity among normal cells, morphologic heterogeneity has long been recognized by pathologists in tumor cell populations fixed and stained immediately upon their removal from the body [9]. In recent years it has been established that tumor cells are also heterogeneous for other characteristics such as the capacity to metastasize, resistance to drugs, and antigenic makeup [10–12]. These observations suggest either that a) tumor cells in a cancer patient have lost their capacity to respond to the hierarchical controls of the tissue and organism; or b) the hierarchical control systems have themselves become ineffective; or c) some combination of a) and b) has occurred. Making a distinction between these alternatives is central to our understanding of the cancer problem and requires a detailed analysis of biological heterogeneity. This analysis may in turn deepen our understanding of normal development, generating a more expansive view of heredity than the one currently held.

Molecular heterogeneity

A graphic example of molecular heterogeneity among normal cells was provided in a clonal analysis of human genital fibroblasts [8]. Cells were cloned from small explants of the foreskin of an individual male, and the activity of 5α-reductase, a microsomal enzyme that converts testosterone to dihydrotestosterone, was determined. The activity in different clones from the same explant varied from the limits of detectability to very high levels.

Clones with low activity gave rise to subclones of low activity, whereas clones with high activity gave rise to subclones with either high or low activity. From these results, we conclude that there is a large variation from cell to cell in the rate of enzyme production. The activity of 5α-reductase is high on the average in uncloned genital skin fibroblasts and undetectable in non-genital skin fibroblasts. Clonal heterogeneity was demonstrated with another microsomal enzyme, adenosine de-aminase, which is found equally in genital and non-genital fibroblasts, but its activity in genital fibroblasts varies independently of the activity of 5α-reductase. Clone to clone variation was noted in another genital fibroblast enzyme, 17β-hydroxysteroid dehydrogenase, and again no correlation was noted with 5α-reductase activity. Given the large number of enzymes in a cell, and assuming that many vary independently of each other, the reality of Elsasser's view that cells exist in a vast number of quantum states [1, 13] is made graphically concrete.

Roger Williams reviewed the evidence for differences in chemical composition among humans in his remarkable book, *Human Individuality* [14]. The evidence is obtained from the composition of blood and tissues for inorganic substances, amino acids, vitamins, and other materials. Inter-individual variation of 2- to 20-fold for each class is commonly found. In the case of bone, which is usually thought of as uniform and homogeneous, the range of apatite values is 5.7-fold for males 20–39 years of age, and 3.9-fold for females in the same age grouping. Differences of 10- to 50-fold in enzyme content of tissues are found even when individuals are matched for height, weight, and total metabolism. Despite the similarities in large-scale characteristics, the details of metabolism can vary 10-fold or more. Williams' data thus provide support for both chemical heterogeneity and the dominance of higher levels of organization over low levels.

Peterson found that clones of hepatoma cells or of hybrids between hepatoma cells and fibroblasts varied over a 30-fold range in their rates of albumin synthesis [15–17]. There was also variation in albumin synthesis among subclones of individual clones, although it was not as pronounced as the variation from clone to clone. Variation was seen in normal hepatocytes within 24 hours of their initiation in cell culture [18]. Immunochemical staining technique showed that there was variation in the proportion of cells producing albumin *within* individual colonies of cells, and also in the amounts of albumin produced per cell. Similar findings were made for the distribution of a surface antigen in cultures of normal and malignant human breast epithelial cells [19]. The estimated rate of variation was somewhat higher in the malignant than in the normal breast cells, but the rates for both were some four orders of magnitude higher than that of conventional mutations. This leaves little doubt that the variation is phenotypic rather than genetic. Peterson believed that the rate of albumin production varied in a $\sqrt{2}$-fold geometric progression. He concluded that there was a probability of between 0.1–0.3 that at each cell division the rate of transcription of the albumin gene on the newly synthesized chromatid would be different from that of the old chromatid [17]. If it were different, it would be half or twice the level of the old chromatid, and the change could occur in either an upward or a downward direction. Up to a 10-fold variation could already be seen *within* individual colonies at the 16-cell stage. Although there are grounds to doubt the quantal nature of the differences in albumin production among cells – i.e., the variation might very well be continuous – there can be no doubt that the rate of phenotypic variation is very high. If the rate of variation in albumin production is representative of that of other proteins, and if those rates are independent of one another, as the data on enzyme production indicate [8], there is little reason to doubt that at any given moment each cell is unique in the details of its molecular constitution.

Although there is great variability in albumin content of normal hepatocytes 24 hours after their removal from the rat to cell culture [18], there is a highly uniform distribution of albumin among hepatocytes in the intact liver of the rat when precautions are taken to avoid loss of the albumin [20, 21]. Within 72 hours of explantation for cell culture the albumin content drops dramatically in all cells to near background levels, although it sometimes remains measurable for up to 10 days [18]. The results

indicate that the uniformity and continuity of albumin production in the intact liver is maintained by the three-dimensional architecture of the liver in the regulatory environment of the organism. When this architecture and environment are disrupted by dispersing the cells for monolayer culture, albumin production decreases, and it does so at different rates among the cells, thereby creating heterogeneity. The hepatoma line used for long-term culture, however, has obviously maintained albumin production over a long period of time in culture, and continues to show great cellular diversity in doing so [18]. It is interesting to note that the coefficient of variation for a tissue-specific antigen is about the same in fresh cultures of breast cancer cells and epithelial cells peripheral to the cancer, but is significantly lower in epithelial cells from normal breasts [19]. Thus, the cells peripheral to the tumor, which are thought to be normal, may have been partially altered by the same conditions that led to the tumor, and may in turn contribute to progression of the tumor by accentuating the abnormal environment of the tumor. The heterogenizing effect of cell dispersal on albumin production in normal hepatocytes recalls the similar effect produced by cell dispersal on cell size of chick embryo fibroblasts [4]. It suggests that the classical pleiomorphism of cells in a tumor may result as much from a diminution of hierarchical controls as on intrinsic defects in the tumor cells. However, since the two levels are interdependent, separation of cause and effect is problematic.

An increase in biochemical heterogeneity was found during progression of medullary thyroid cancer in humans from the earliest premalignant hyperplasias and microscopic carcinomas to the metastatic stages [22]. In general, the distribution of calcitonin was reasonably homogeneous in palpable primary tumors. However, a small subset of individuals had primary tumors with a heterogeneous distribution of calcitonin among cells, similar to that found in metastases. Five of the six patients with a heterogeneous distribution died within 1 to 5 years of removal of the primary tumors. In contrast, all of the 11 patients with a homogeneous distribution of calcitonin were alive during a similar follow-up period. The implication of this finding is that the loss of capacity to maintain a uniform composition and behavior of thyroid cells is correlated with increasingly malignant behavior of the cells. It emphasizes once again the importance of investigating the role of hierarchical control in the development of malignancy.

Functional changes

Protozoa

In the early years of this century Victor Jollos, a German protozoologist, found that cultures of Paramecium *aurelia* would adapt to a variety of deleterious treatments [23, 24]. The treatments included arsenic, high salt concentrations, heat, and antiserum to surface antigens. He showed that there was adaptation by individuals rather than selection of spontaneously occurring genetic mutants, with the evidence indicating cytoplasmic rather than nuclear control. Since these adaptive changes persisted for many generations after the return of the Paramecium to normal conditions, Jollos called them *Dauermodifikationen*, or enduring modifications. His work was taken up in this country by Tracy Sonneborn, a leading protozoan geneticist. He confirmed that resistance to antiserum specific for surface antigens was indeed adaptive, but had selective features [25]. About 20% of the individuals in the population he started with were *transiently* resistant to the antiserum. The transient nature of the original resistance was apparent in experiments in which he started with a number of individuals, and allowed each to undergo one vegetative division to two sister organisms. He then exposed one of each pair to the antiserum, and allowed the other to multiply to form a large clonal population. The individuals that survived antiserum treatment were removed from antiserum, and yielded clones that were resistant. However, the clones formed from the unexposed sisters of the resistant cells had the same high sensitivity to antiserum as clones from sisters of cells that had been killed by the original antiserum. Since at least some sisters of

resistant cells were themselves presumably resistant, the sensitivity of their clonal progeny showed that the resistance was transient, indicating that the population was always heterogeneous and was constantly generating phenotypically resistant cells, whose descendents would ordinarily revert to sensitivity if not exposed to antiserum. Exposure to the antiserum, however, appeared to fix the transiently resistant cell in that state so that its descendants remained resistant for as long as 300 generations after removal of the antiserum. Resistance to antiserum was abrogated by mating resistant cells either to non-resistant cells, or to other resistant cells. Indeed, the cell would lose its resistance even if it underwent autogamy, a process in which the cell undergoes the physiological changes of mating without actually mating – clearly indicating the non-genetic nature of the phenomenon. Thus, Dauermodifikationen were the first verified cases of the selection of states or metabolic patterns as opposed to genetic mutations. They illustrate the basic importance of heterogeneity in adaptive phenomena, a subject which will be further considered below.

Plant cells

Plant cells have one distinct research advantage over animal cells, namely the capacity to regenerate whole plants from clones of somatic cells. This capacity has been used to advantage in studying the adaptation of plant cells in culture to multiplication in the absence of a cell division factor [26]. Tobacco pith cells require a cell division factor or cytokinin to multiply in culture, but may become habituated to multiply without it. The habituation process is a gradual one which leads to progressively more habituated tissue. Clones isolated from the same line of habituated tissue varied widely in growth rate without cytokinin. Clones with either a low or a high capacity for multiplication without growth factor were used to derive subclones. There was extensive variation among the subclones in their capacity to multiply without the growth factor. Most of them differed from their parent clone in this capacity, but their mean values were biased toward those of the parent clones; i.e., the high capacity clones tended to give rise to high capacity subclones, etc. It was concluded that individual cells differ in degree of habituation, and that they can shift to higher or lower states of habituation. When a slightly habituated clone was grown without the cell division factor over a period of 2 years, its degree of habituation gradually increased. Subclones isolated from the parent tissue at various times had, *on the average,* the same degree of habituation as the parent population. Although the subclones showed wide variation in the degree of habituation to growth without cytokinin, the average increased with time, reflecting progressive increases in the degree to which individual cells were habituated.

Habituated clones could be regularly induced to form leafy buds that later developed into complete fertile plants. The capacity to form leafy tissue, which is a measure of their organogenic potential, was *unaffected* by the degree of habituation. Since the cells of leafy tissue which developed from habituated clones reverted to a requirement for cell division factor, there was clear evidence that habituation was a result of epigenetic rather than genetic change. It appears that the epigenetic change occurred gradually through a continuous process of selection among populations that were continuously generating phenotypic variants. Thus, heterogeneity provides the phenotypic variation required for adaptive change.

Animal cells

As noted earlier, the first explicit evidence of animal cell heterogeneity, and particularly of the effect of the environment in fostering heterogeneity, was obtained in measuring the size of mesodermal cells cultured directly from chick embryos [4]. Two hours after the cells were placed in culture, those which had attached to the dish were found to have a fairly narrow distribution of cell sizes. In addition to the generation of size heterogeneity already mentioned, the average protein content per cell was found to vary in proportion to size. The change

in both characteristics was retarded by omitting serum from the medium, which has the effect of blocking further growth and multiplication. It is evident that organization of cells in a tissue limits their average size and the distribution of sizes.

Studies on the number of divisions human cells can undergo in culture before they senesce reveal great heterogeneity among cells arising from the same donor. Cultures of diploid fibroblasts derived from human fetal lung undergo about 50 divisions before senescing [27]. Individual clones, however, exhibit a wide range of capacity to multiply, with some undergoing very few divisions [5, 6]. Daughter cells derived from the same parental cell frequently diverge widely in the number of divisions they undergo before senescence [28]. Similar observations have been made on the time required for individual cells to divide (interdivision time) [7]. Cinematographic studies show that progeny of the same cells may differ widely in their interdivision time. This is especially true for cells that have undergone many passages in culture in which the overall growth rate has decreased. In some sister-sister pairs of late passage culture, the variation in generation time was greater than 40 hours. In the early passage culture, there is a fairly high correlation in division times between sister-sister pairs, but there is poor correlation between parents and progeny. The study of intraclonal variation in normal diploid cells is complicated by the withdrawal of many cells from the cell cycle. For example, one clone had *some* cells that had undergone 10 divisions. If all of the cells had divided, there would have been 1024 cells, but there was a high dropout rate and the final clone size was only 113.

Detailed microcinematographic studies have been conducted on the growth rate of an established line of cells in low concentrations of serum [29, 30]. They reveal marked heterogeneity in proliferative capacity of Swiss 3T3 cells. This heterogeneity arises at extremely high frequency within a clone, with sister cells often showing considerable difference in capacity for further proliferation. The authors conclude that it is extremely unlikely that the heterogeneity is due to mutation or karyotypic instability because of its extremely high frequency, as well as the fact that most of the cells that cannot divide in low serum concentrations can do so when the serum concentration is increased. They suggest that differences arise between cells because some are unable to sustain production at low serum concentrations of some rate component that is required for the cell to respond to growth factors.

Attempts were made to select a population with a high capacity for growth in low serum or growth factor concentrations, but these were unsuccessful, indicating that the differences among the cells were not stably inherited [31]. In another study, using NIH 3T3 cells, the growth rates of clones differed widely during their development from single cells, even in high concentrations of serum [32]. The growth rates of subclones obtained from individual clones also differed widely. However, subclones derived from slower-growing clones tended to multiply faster than the parental clone, while the opposite was true for subclones derived from faster growing clones. Such median-directed growth behavior again indicates the transient nature of the differences in growth capacity of cells, and accounts for the constancy of growth rate of the population as a whole. However, as we shall see below (see Transformation and tumor producing variants), prolonged growth of the NIH 3T3 population in a low concentration of serum does indeed result in a stable, adaptively increased saturation density in low serum concentrations [33, 34]. The difference between these cells and the Swiss 3T3 cells described above may be that right from the start the NIH 3T3 cells sustained continuous exponential multiplication up to saturation density in the selectively low serum concentrations, whereas the multiplication of the Swiss 3T3 cells in low serum decreased continuously.

Metastatic heterogeneity

Morphological heterogeneity or pleiomorphism has long been noted by pathologists to be characteristic of the cell populations that make up many tumors [9]. However, the potential significance of tumor cell heterogeneity in neoplastic development was not recognized until it was related to the capacity of cells to metastasize. Such a relationship

was first suspected when it was found that alternating passage of melanoma cells from metastases in mice and in cell culture markedly increased the number of lung metastases in the mice [11]. It was then shown that clones of the melanoma line differed more than 100-fold in their capacity to produce metastases upon intravenous inoculation into mice. On the basis of these and other similar results, it was concluded that cells in tumors differed markedly in their capacity to metastasize, and that metastases originated from those tumor cells with an inherited proclivity for metastasis. However, some workers failed to find that cells from metastases were more metastatic than the parent tumor cells [11, 35]. They concluded that the occurrence of metastasis was the result of a random event occurring among cells in the primary tumor. The differences between the selective and random points of view were apparently reconciled with the finding that the results depended on the tumor cell population studied [36]. Metastasis by unselected, poorly metastatic cells was selective, while metastasis by previously selected lines appeared to be random.

It has been noted that new lines derived from established metastatic lines are not necessarily metastatic [35, 37]. The reversion of metastatic to non-metastatic behavior may occur on passage in culture or, remarkably enough, upon serial selection of experimental metastases. A 'dynamic heterogeneity model' has been invoked to explain the high rate of variation to and from metastatic ability [38, 39]. It is based partly on finding non-metastatic cells in metastases, and on the failure to increase metastatic efficiency beyond a limited value by serially transferring metastatic cells. The model proposes that highly unstable metastatic variants are generated at high rates in the lines studied, resulting in a dynamic equilibrium between generation and loss of metastatic variants. The conclusion that these variants are of mutational origin is based on the observation that the ratio of variance to mean metastatic capacity in sets of clones is high. However, this technique, known as fluctuation analysis, has many problems, some of which have recently been pointed out [40, 41]. One particular problem arises from starting the fluctuation analysis with single cells, since a high degree of phenotypic heterogeneity characterizes these populations, so a high variance is assured. In addition, it has been pointed out that the capacity to metastasize is strongly affected by pretreatment of the cells. For example, growth of B16 melanoma in spheroids for 3 days instead of attached to a dish increased their capacity to metastasize more than 20-fold; returning them to the attached state for 1 day reduced their metastatic capacity to the original value [42]. Inoculating cells into mice together with plastic microspheres greatly increased the number of metastases formed by poorly metastatic clones of a sarcoma line, and eliminated the difference between them and highly metastatic clones [43]. There is, in addition, the problem of using Poisson statistics to analyze interclonal differences with an assay that shows large deviations from a Poisson distribution in assaying a single clone for metastasis in a group of mice [43, 44].

Part of the conclusion about the mutational origin of the metastatic variants comes from an observed increase in their proportion with an increase in the size of the cell population. However, at the same time there is a 2.4-fold increase in population density of the larger population, despite a claim to the contrary [38]. Since spontaneous transformation [33] and tumor formation [45] are markedly increased as a function of cell population density, the increase in population density seems a likely source of the increased metastatic capacity. The latter could not, then, be taken as evidence for the mutational origin of the metastatic variants. It is more likely that they are phenotypic variants of the type discussed for many other cellular properties in this article. Such variants have a wide range of stabilities, which would explain the failure to observe increased metastatic capacity of cells from some metastases.

Cell surface antigens

The study of cell surface antigens has two distinct advantages. Cells bearing a particular surface antigen can be killed by specific antibody in the presence of complement, leaving non-antigen bearing

cells as scorable survivors. They can also be labeled with fluorescent antibody or lectin without damaging the cells. The cells can be sorted by fluorescence-activated cell sorting, and grown to large populations for further testing. The first methodology was used to demonstrate in mice that both normal thymocytes and leukemia cells bearing a thymus-leukemia specific antigen (TL^+) lose the antigen if the mice are treated with antibody to the antigen [46]. This also occurs in thymocytes of TL^+ mice suckled on mothers highly immunized against TL, but returns after weaning. The suppression of TL antigen was termed antigenic modulation. Modulation could be produced in cell culture with TL^+ leukemia cells by treating with antiserum [47]. It was detectable within 10 minutes and complete within 1 hour. Modulation was prevented by inhibition of RNA synthesis or incubation at 0° C during the serum treatment, indicating that it is an active process. After removal of antibody, some return of antigen is detected in 24 hours, and it is complete in 7 days. Unlike the *in vivo* case, modulation does not occur in normal thymocytes in culture, but this may be related to their failure to multiply and perhaps to carry out active metabolism. Modulation of the TL antigen bears certain similarities to the long-lasting modification of surface antigen in Paramecium, except that its appearance and disappearance are much faster.

Subsequently, cells of a metastasizing, chemically-induced lymphoma were found to lose their transplantation antigen in adapting to a T-cell mediated anti-tumor immune response [48]. This happens during metastasis of immunosensitive cloned tumor lines in a normal immunocompetent host. In contrast to antigen modulation, this antigenic change is stable, and inherited for over 100 tumor cell generations. It occurs with high frequency and within three generations of cell multiplication after inoculation into the mouse. The variants did not exist in the original population, and could not have been selected from it. This stable change appears to be a response of cells to their microenvironment, consisting of an adaptation to a T-cell mediated anti-tumor immune response, and not to the selection of preexisting mutants. The lymphoma was found to become more invasive and metastatic when it lost its tumor-associated transplantation antigen, suggesting that the change in metastatic behavior was related to the reduced susceptibility to the immune response. A better understanding of changes in surface antigens, therefore, should improve our understanding of tumor progression and associated metastases.

Background for understanding the adaptive changes to antibody has come from recent studies using flow cytometry. There is general agreement that tumor cell populations in culture exhibit considerable heterogeneity in the expression of surface antigens [49, 50]. The heterogeneity of any one antigen is independent of the variation of the others. Cells that are selected for high or low concentrations of antigen maintain that characteristic when reanalyzed immediately. Clonal isolates of myeloma and lung tumor cells tend to have as much heterogeneity for antigen as the parental population, and most of them have about the same average amount of antigen [49, 50]. This indicates that most of the variation is nonheritable. However, a few clones have distinctly different amounts of antigen, and maintain the difference on passage. In more recent studies of bladder carcinoma lines, sorting of cell populations by antigen expression gave cultures with heritable antigenic differences that persisted for weeks [51]. Of 64 clones obtained from cells that had been isolated from the low end of the scale of antigen expression, most resumed the distribution of antigen found in the parental line, but 19 continued to express low amounts of antigen.

When fractions of myeloma cells were isolated with low and high antigen expression, they drifted back to the parental levels in 7 days [49]. However, cells with little or no antigen were temporarily immune to killing by antiserum during the time they were in that state. Variations in one antigen occurred independently of variations in other antigens. We conclude that clones rapidly generate great heterogeneity in antigen expression, and that most of the variants are temporary. However, a minority of the variants are stable, with the proportion depending on the particular system under study. It was possible in two successive selective steps of a low antigen fraction to stably increase the

fraction of low antigen cells from 15% to 45%. It is of interest that patients with non-invasive bladder carcinoma are more likely to suffer recurrence and invasion if their tumors abnormally express two antigens than if they express only one [52].

Drug resistance

It is well-documented that subpopulations and clones of cells from the same tumor exhibit heterogeneity in sensitivity to drugs [10]. This is an obvious source of problems in chemotherapy of cancer. Perhaps the most extensive evidence among animal cells for heterogeneity in drug sensitivity, however, has come from an investigation of the development of resistance to bromodeoxyuridine of a near-diploid established line of Chinese hamster cells [53]. As the cells develop resistance to bromodeoxyuridine resulting from long-term passage in low concentrations of the drug, they also lose, to varying degrees, their capacity to multiply in a selective medium containing hypoxanthine, aminopterin, and thymidine (HAT medium). After 9 weeks of passage in bromodeoxyuridine there is a 10^5-fold variation among clones in their capacity to multiply in HAT. While the HAT test is, in a sense, a measure of sensitivity to aminopterin under special conditions, it is a useful indicator of the enormous variation in drug sensitivity. It could also be shown that almost every clone from the original culture passaged in bromodeoxyuridine loses its capacity to grow in HAT medium at a different rate from the other clones; some of the clones retain their full capacity to multiply in HAT despite serial passage in bromodeoxyuridine for 22 weeks. Indeed, subclones obtained from a single small colony of 100 to 200 cells varied in the rate at which they lost the capacity to grow in HAT medium, which clearly shows that variants are continuously generated at a high rate. The high rate of variation in drug resistance characters suggests that growth in low concentrations of a drug could select phenotypic variants, and that these could be stabilized by continuing selection of a continuously varying population. Evidence was obtained that a large fraction of the bromodeoxyuridine-resistant, HAT-incompetent population could be returned within a few days to its original bromodeoxyuridine-sensitive, HAT-competent state by treatment with 5-azacytidine, butyrate, or ethionine [54]. This massive, short-term reversion, along with the extreme heterogeneity, showed that the drug-resistant state was the result of phenotypic rather than genotypic change.

An earlier set of experiments [55] gave strong evidence that the development of mammalian cells resistant to purine analogues by exposure to low doses of the drugs was adaptational in nature. Resistance could be obtained under conditions of little or no toxicity to the population of cells. First step resistance was induced in 2 to 3 days and was lost when the cells were grown in the absence of selective pressure for 6 to 9 weeks. Continued growth in low doses of purine analogue resulted in progression to higher levels of resistance. It was concluded that the resistant lines were non-mutants that resulted from drug-induced modifications in cellular biochemistry.

Transiently resistant colonies also arise in the presence of low concentrations of purine analogues [56–58]. When retested, cells from these surviving colonies display the same high sensitivity to the analogues as the original unexposed culture. This shows that phenotypic heterogeneity for resistance exists among cells of the original population. The transiently resistant cells are likely to be the ones that give rise to more stably resistant cells upon prolonged exposure to the drugs, in analogy to the Dauermodifikationen of *Paramecium* [24, 25].

Malignant cell lines made resistant to a single chemotherapeutic agent by stepwise incubation in increasing amounts of the drug have been found to be resistant to other, structurally unrelated cytotoxic compounds [59]. This phenomenon is called multidrug resistance (MDR). It is thought to be related to some cases of drug resistance in chemically treated cases of cancer. Most cell lines with the MDR phenotype show increased expression of the gene encoding P-glycoprotein, termed the *mdr* gene. It is thought that the P-glycoprotein is an energy-dependent drug efflux pump. There is, in fact, evidence that contradicts the idea that the P-glycoprotein is responsible for MDR, including

he observation that resistant cells can withstand a nuch higher *intra*cellular concentration of drugs han that required to kill sensitive cells. It is, how-ever, considered the defining marker for 'classical' MDR, despite the finding of increasing numbers of atypical' MDR cases. An interesting correlation is noted between the development of MDR in some cultured cell lines and the response of liver cells *in vivo* to treatment with carcinogenic chemicals. Such treatment results in the appearance of many microscopic foci of altered cells in the liver, which develop into nodules of multiplying cells [60]. The foci are heterogeneous in their composition of several enzymes. The nodules are resistant to a variety of compounds, and consequently have been named resistant hepatocyte nodules. They therefore exhibit the MDR phenotype. An outstanding characteristic of the resistant hepatocyte nodules is that more than 95% of them remodel into normal liver tissue once administration of the carcinogen has ceased. The nodular response is therefore considered a physiological adaptation by the liver to cytotoxic compounds, and indeed 100% of nodule-bearing rats survive doses of CCl_4 that kill all the control rats. The functional relationship between MDR and resistant hepatocyte nodules raises the question of whether MDR is also an adaptive response, rather than the selection of a mutation which results in overexpression of the 'classical' *mdr* gene. There is, of course, the additional evidence that resistance established in cell lines to metabolic analogues is reversible and basically adaptational in character [44, 54, 55]. Therefore, we again have a suggested relationship between heterogeneity and a non-mutational, adaptational response, which will be considered further below.

Cell multiplication

Most transformed, tumor-producing cell lines initiate the formation of colonies in agar, and the colonies are highly variable in size. This was true of cells from a cloned line of spontaneously transformed Balb 3T3 cells, but the colonies did not breed true: both small and large agar colonies gave rise to cell populations which produced a broad range of colonies when reseeded in agar [61, 62]. The variations in colony size, and therefore growth rate in agar, were indicative of transient fluctuations in the population. Sublines derived from morphologically non-transformed and transformed clones of the Balb 3T3 line had different rates of multiplication and saturation densities in culture, and the transformed sublines differed from each other in their capacity to produce colonies in agar [63]. Although only the transformed sublines produced colonies in agar and tumors in mice, there was no quantitative correlation between these two properties among the transformed sublines, nor with the rate of glucose utilization, which also varied among all sublines. Cells derived from the mouse tumors had agar colony forming efficiencies that were different from those of the parental lines used to initiate the tumors. The results indicate an enormous capacity for variation in the growth and metabolic capacities of the cell line.

Some indication of how quickly variations in growth capacity can arise was obtained by subcloning a newly transformed agar colony of Balb 3T3 cells [64]. Four of the five subclones had subtle but persistent morphological differences, while the fifth had a strikingly different morphology. The subclones differed in repeated assays for colony formation in agar and in tumor producing capacity in nude mice, and there was no correlation between those characteristics. However, similar studies done with a second newly transformed agar colony produced subclones that were not recognizably different in appearance from one another, and all had about the same high efficiency of colony formation in agar. The results indicate that variation may occur at a very high rate in some clones but not in others. In other words, there is even heterogeneity of the capacity to undergo variation.

The subclones from the variable clone [64] were studied over a 6-month period [65]. During that time they tended toward a common morphology, and toward a common high colony-forming efficiency in agar, although one subclone remained consistently higher than the others in this respect throughout the entire period. Differences in tumor forming capacity remained after 18 weeks in passage, although the subclone that produced the most

rapidly growing tumors in the assay at 4 weeks produced the slowest growing tumors at 18 weeks. No statistically significant differences in chromosome number were found. The long-term studies in culture indicate a trend toward a common morphology and behavior in culture, but a perpetuation of large differences in tumor forming capacity; i.e., the cells develop a similar growth capacity for the medium in which they are growing, but not for a completely different environment. It is apparent that selection for growth under one condition is not closely coordinated with selection for growth under entirely different conditions.

A series of papers described tumor formation in nude mice by serial dilutions of spontaneously transformed Balb 3T3 cells, the changes which occurred in the capacity for multiplication in culture during development of the tumors, and a clonal analysis of those changes [66–69]. The behavior of the cells, and particularly of individual clones, was extremely complex, and only an overview of the major trends can be recounted here. However, they are included because they show conclusively that physiological adaptation makes a large contribution to the development of competence to grow in radically altered environments. As expected, the latent period to tumor formation varied inversely with the number of cells inoculated. It took 10^3 to 10^4 cells to initiate progressive tumor formation in the nude mice. The tumors were mainly primitive, pleiomorphic sarcomas, although some were a little better differentiated than the others. In general, the cells derived from the tumors grew more slowly in culture than the cells used to initiate the tumors, but this was especially marked in cells from tumors with a very long latent period following the inoculation of small numbers of cells. Clones of cells derived from all the tumors often varied greatly in growth properties, even when the tumors were initiated by recently cloned cells. The diversity was most marked when measured by colony formation in agar, and was most apparent in the first assay carried out after tumor removal and clonal isolation. Clones derived from tumors that arose from fairly large inocula tended to show the same pattern of improved growth with time in culture as the uncloned tumor cell populations, but there were cases of individual variation in pattern. In some cases, no tumor cell clones had a growth rate in culture that matched that of any of the clones from the cells used to initiate the tumor. After prolonged culture of the uncloned tumor cells, they attained growth rates and efficiencies of colony formation in agar that were higher than those of any clones obtained directly after removal of the tumors. These results suggested that the evolution of the transformed cell line to a tumor in the mouse and the evolution of the tumor cells to improved growth in culture involved more than just the selection of pre-existing variants; that is, adaptive changes were involved in responding to the new environments. They also indicated that the adaptive growth of cells in a mixed population was more than the sum of their growth as individual clones.

Clonal variation was most extreme in cells from the longest delayed tumors: when 40 to 55 days elapsed before tumor growth became detectable, then the tumors remained at constant size for several weeks before they entered a period of rapid growth [68]. Although these tumors were growing rapidly in mice at the time of their removal, and had a high labeling index with ^{3}H-thymidine, fewer than 1 cell in 10,000 could initiate colonies in culture, compared with the 50% cloning efficiency of the cells used to initiate the tumors. The growth rates of the few cells that could multiply in culture were generally low, but extremely varied. Some of the clones lost the capacity for further growth in a few passages. Others persisted, but their growth rates fluctuated unpredictably in early passages. The uncloned population showed a steady increase in growth rate, which may have resulted from selection of the more rapidly growing clones. One individual clone maintained an extremely low growth rate in weekly passages for 5 months, and then showed a stepwise increase, suggesting the appearance and selection of a faster growing variant. Even after this increase, however, this clone multiplied at a considerably lower rate than the original cultured cells used to initiate the tumor. It seems fair to say that the progeny of each tumor cell that could grow in culture differed from the progeny of every other one in its initial growth rate and subsequent history of growth in culture. The heterogeneity of

growth rates in culture of the cells from the delayed tumors was far greater than that of the growth rates in culture of the cells used to initiate the tumors. Even the fastest growing clones from the delayed tumors grew more slowly in successive assays in culture than the slowest growing clones from the population used to initiate the tumors.

In order to study the nature of the changes in capacity for growth in culture of slow growing clones from the delayed tumors, a clone that was taken directly from the tumor was itself subcloned directly upon its isolation. The original clone grew at a constant low rate for 3 weeks and then fluctuated in growth rate for 4 weeks before beginning a steady increase to a fairly high growth rate, where it remained during four additional months of repeated passaging. Three of the 20 subclones failed to multiply further after their initial growth to a full colony. The growth rates of six of the slower growing subclones were measured repeatedly during successive weekly passages. The growth rates of all of them increased steadily and at about the same rate over a 6-week period before reaching the same constant rate of growth. The synchrony of the rise in growth rate of all the slow growing subclones over a 6-week period indicated that the increase represented a physiological adaptation of the tumor cells to tissue culture growth conditions.

An experiment to provide a distinction between selection of spontaneously occurring mutations and physiological adaptation was done with one of the slow growing subclones just described. The cells were passaged either at extremely low density or at 100 times higher density with varying frequencies of passage and feeding. The experiment was designed to observe the effect of cell number and of physiological state on the increase in growth rate with time. The increase in growth rate occurred independently of the number of cells used in passage, but it was dependent on the passage and feeding frequency of the high density seedings; that is, the more active the metabolism and growth, the faster the increase in growth rate. The results therefore reinforced the conclusion that the progressive increase in growth rates represented physiological adaptation rather than the selection of spontaneously occurring mutants. However, this left us with no clear indication of how adaptation occurred. One suggestion, however, which arose from the great clonal heterogeneity in growth rate of the cells from the delayed tumor, was that fluctuations in *phenotypic* growth rate would permit selection of phenotypes (rather than genotypes) capable of faster growth, and that successive phenotypic selection would result in stabilization of the improved growth. This process of adaptation through successive selection of phenotypic states will be considered further in the section below on transformation and tumor producing variation, in which the concept of progressive state selection is developed.

Possibly the most extreme example among these cases for the generation of growth heterogeneity was uncovered during a routine assay of a clonal line of non-transformed Balb 3T3 cells for colony formation in agar [70]. Seven small colonies and two large colonies appeared following the seeding in agar of 10^5 cells. When these were isolated and cultured on plastic, the cells of each subclone were morphologically distinguishable from the cells of the other subclones. The subclones varied from each other over a 3-fold range in growth rate on plastic, and about 1000-fold in efficiency of colony formation in agar. This discrepancy indicates that the extent of observable heterogeneity in a population depends on the measurement used, with the more demanding assay (growth in agar) exhibiting the greater degree of heterogeneity. Immediate subcloning in agar of cells from one of the two large agar colonies gave rise to 13 agar colonies, 9 of which declined sharply in growth rate to the point of extinction in a single passage on plastic. When first assayed, one of the remaining four colonies had a much lower capacity for growth in agar than its three surviving compatriots, although it caught up in later passages.

The overall results revealed that the growth behavior of each clone from the original line was unique, and showed again that intraclonal progeny can become different during the initial development of the colony. It was of particular interest that the extreme transformation found in two of the nine clones occurred only after the initial seeding of cells in agar, but not when the cells were grown

exclusively on plastic, although it would have been easily detected there. The increased propensity for transformation under the constraint of growth in agar suspension had been observed before [64], and occurred again several times in the course of this experiment, suggesting that moderate growth constraint elicits transformation as part of an adaptive response to the constraint.

Given the high degree of phenotypic variation in growth capacity, one might expect that there would be a continuous selection for those cells with the highest growth rates, and further variation would be reduced or minimized. This was, in fact, realized with the explantation of cells from delayed tumors, where there were enormous differences in the initial growth rates of clones. In that case, the environment of the cells was changed from the subcutaneous tissue of the animal to the floor of a culture dish. What happens among cells kept in a more or less steady state in culture for extended periods of time? A wide range of growth rates on plastic was found among clones isolated from the NIH 3T3 line [32]. Single cells were isolated from clones, and the growth rates of the developing subclones were determined from the single cell state onward. There continued to be a wide range of growth rates among progeny of individual clones. Offspring from fast growing parents rarely exceeded the growth rate of the parents. It was concluded that the overall growth rate of NIH 3T3 populations includes continuous variations in growth rates of individuals in the population. Population growth rates remain stable because the fastest growing parents generally produce offspring that multiply more slowly than the parents, while the slowest growing parents tend to produce offspring that multiply faster. It seems likely, however, that the continuous application of growth constraints such as reduction of serum concentration would select those phenotypes better able to grow under the constrained conditions and thereby change the character of the population.

Transformation and tumor producing variants

The human epidermal carcinoma line (HEp-3) grows on the chorioallantoic membrane (CAM) of the chick embryo and metastasizes to the organs of the embryo [71]. Upon serial passage in cell culture, there is a progressive loss of metastatic potential and of tumorigenicity [72]. Clonal analysis using cells isolated directly from CAM tumor revealed that many clones lose the potential for tumor production within the 22 to 25 cell generations required to produce a suitably large population for re-injection onto the CAM [73], although the cells were obviously growing as part of the CAM tumor when they were first isolated. The other clones produced tumors of markedly different sizes when they were first cloned, but all lost the ability to initiate tumors after 40 cell generations in culture. The tumor cells that gave rise to clones that failed to produce tumors after 22 to 25 generations did not multiply faster in an enriched medium (F12) when first isolated than those that required many more generations to lose the capacity for tumor formation. Hence there was no indication that growth in culture resulted in the selection of cells that were genetically endowed with a superior capacity for multiplication in culture and an inferior capacity to multiply on the CAM. It should be noted, however, that the cells that failed to give tumors multiplied in culture at a much faster rate than those that did give tumors, when grown in a medium (DME) less complex than the F12 medium used in the clonal experiments. It is apparent, therefore, that the cells that made up the tumor were a heterogeneous lot, losing their capacity for tumor formation on the CAM at widely different rates when placed in culture. Another aspect of their diversity appeared after the clones had lost the capacity for tumor formation. While this capacity could be restored in all clones by serial passage on the CAM, the time required for restoration varied from 3 to 12 weeks. There was no evidence that the cells that failed to produce CAM tumors multiplied at all on the CAM. However, the capacity to produce tumors could be restored by repeated *in vivo* passaging of the inoculated CAM, beginning with inocula as small as 1000 cells. Indeed, the number of CAM passages required to restore tumorigenicity decreased only slightly when the initial inoculum was increased 100-fold. It was con-

cluded that both the loss of tumor-producing capacity during cell culture and its restoration during passage on the CAM *reflected an adaptive response* by the cells to their physiological environment. Although the selection of conventional spontaneous mutations was effectively ruled out, the prevalence of clonal heterogeneity indicates that the cells were continually undergoing phenotypic variation. This raises the possibility of progressive selection of fluctuating phenotypic states.

The NIH 3T3 line of mouse embryo cells is widely used as an indicator for transformation by transfection with certain cellular oncogenes. However,it readily undergoes spontaneous transformation when subjected to certain conditions, such as maintenance in the confluent state for a week or more or repeated passage at low population density in low serum concentrations [33]. (Transformation is defined here as the capacity to produce foci of multiplying cells on a background of non-multiplying cells under a defined set of conditions.) Alternatively, the cells can be maintained for many months in the non-transformed state by frequent passage at low density in high serum concentration. The results indicate that conditions of moderate contraint on metabolism and/or growth evoke an adaptive response in some cells of a population, which allows them to function more efficiently under the constraining conditions. There is evidence for an increase in the growth capacity of many of the cells in the population, in addition to the cells that produce foci [34]. Clonal analysis shows that all of the cells in a population exposed to the constraining conditions have an increased capacity for focus formation. However, there is a very wide range in this capacity among the clones, and it changes with repeated passage of many of the clones. No correlation was found between the capacity of these clones for focus formation and their growth rate under non-constraining conditions (unpublished observation). Both the phenotypic heterogeneity among clones in focus formation and the change with time in focus forming capacity within clones suggest that conditions of moderate constraint may select those phenotypic states capable of functioning most effectively under those conditions. A similar effect of cultivation at high cell density in promoting spontaneous transformation or metastatic capacity of other lines of mouse cells has been described [45, 74, 75]. Although no data are given in these articles for heterogeneity of the capacity for transformation or tumor formation in the original population, it is reasonable to infer its existence on the basis of other work [64, 70, 73]. This, of course, raises the possibility that progressive state selection has general significance as a model for cell transformation.

There are also precedents for the finding that the tumorigenicity of the human epidermal carcinoma can be reduced or eliminated by growth in culture [73]. For example, hamster cells transformed by infection with polyoma virus are returned to their non-transformed appearance and behavior by growing them at very low density [76, 77]. A similar finding was made with mouse embryo cells transformed by x-irradiation [78, 79]. The normalizing effect of conditions for maximum growth and metabolism were borne out by the finding that spontaneous transformation of NIH 3T3 cells associated with moderate growth constraint could be reversed by growth at low density in high serum concentrations [34]. It is possible that there is selection for a state of more regulated function under conditions of maximal stimulation, and therefore that phenotypic heterogeneity plays a role in reversion as well as induction of transformation.

Population changes under constant conditions

Emphasis up to this point has been placed on heterogeneity at the cellular or clonal levels, and its relation to population-wide changes under selective conditions. It is a fairly common experience that population-wide changes occur in cellular properties despite attempts to maintain constant conditions in cell culture. This type of event is usually considered a nuisance in the sense noted in the quotation from Elsasser that introduces this article, and it either goes unreported, is rejected for publication, or remains unread. This is, no doubt, because the detailed observations are by nature difficult or impossible to reproduce with precision and because they cannot be reduced to

molecular mechanisms, although they are, in Elsasser's thought, essential to an understanding of life. Therefore, I will report some published and unpublished examples of my own, and consider some others.

A large stock from the same passage of Balb 3T3 cells was distributed in vials and kept in liquid nitrogen for about 1 month after their spontaneous transformation, but a line of the same cells that was not frozen was kept in continuous passage. At various times, a vial of the frozen cells was thawed and passaged in parallel with the non-frozen line. The morphology of the various sublines was noted, their growth rates and tumor producing capacities were determined, and every other week for almost 3 years, their capacity to form colonies in agar was measured [63, 80].

Cells of the first two consecutive thaws differed morphologically from each other and from the non-frozen population [63]. The thawed populations multiplied in culture at different rates, but both were consistently slower than the non-frozen population [63, 80]. All three sublines produced tumors in nude mice and glycolized at different rates [63]. Another two samples were later thawed [80]. All five sublines had different capacities to form colonies in agar in early assays, varying from less than 1% to about 70%. One of the sublines failed to continue multiplying after about 30 weeks in culture, and another was approaching such a low point of growth after 30 weeks that it was terminated. After many passages in culture, the other two thawed populations gradually approached the high capacity for colony formation in agar of the non-frozen sample. It is clear that despite the common origin and handling of the cells, they differed in many properties, and they underwent radically different evolutions over many passages. However, freezing of the previously non-frozen population more than 100 passages after its transformation gave a stock that behaved reproducibly on thawing [80]. Apparently the cells were sensitive to the effects of freezing shortly after they had become transformed, but not later.

Another account of change in behavior with time has been reported from Nicolson's laboratory [81], using cell clones from early and late passages of a clone from spontaneous rat mammary adenocarcinoma. The clones were relatively homogeneous in growth rates and saturation densities, but differed from one another in morphology, metastatic potential, marker chromosomes, and modal chromosome number. Phenotypic diversification occurred at disparate rates for different phenotypic properties, so that the clones became heterogeneous for most of the properties measured. There was simultaneous independent divergence of several characters in the progeny of a single cell, resulting in a mixed cell population, and each population differed from the other clonal populations, which were all derived from the same original clone. If there ever was confirmation of Elsasser's dictum that every cell is a unique individual, this is it.

I have observed sudden, unexplained changes in the behavior of cultures. One subline of NIH 3T3 cells that gave rise to only a few foci on a thin monolayered background switched within 1 week to producing a thick monolayer with no foci (Rubin, unpublished). Transformed cells that would form foci when added to the earlier culture no longer did so on the later ones. Once the change in mass behavior occurred, it persisted in later passages. I could cite other instances of sudden unexplained changes in cell behavior with passage under presumably constant conditions, all suggesting that cells are either extremely sensitive to minute and subtle changes in the cellular environment, or that they have a common intrinsic tendency for massive change within a short time. Thus, we have, in addition to variability at the cellular and clonal levels, a variation of whole cultures reminiscent of coordinated, multicellular changes that occur during normal development.

Discussion

Heterogeneity among cells and clones of a population has been clearly demonstrated for many properties of cells in culture and in tumors. The degree of heterogeneity for any particular property depends upon the refinement of the measurement and the constraints of the conditions imposed. For example, little heterogeneity of cell division time is

apparent when cells are kept in a high concentration of serum, but it is evident in growth-limiting concentrations of serum [30]. Transformed cells that show little difference in growth rate on plastic may exhibit large differences in the more demanding capacity to form large colonies when suspended in agar, or may give rise to metastases *in vivo* [70, 81]. If non-transformed clones are being compared, however, no distinction can be made in agar, since the challenge is too severe, and none of them can grow enough in suspension to make a colony. Clones of human epidermoid carcinoma cells taken from an experimental tumor on the CAM vary greatly in their capacity for tumor production on the CAM, as well as in their growth rate in one medium in culture, but not in a more enriched medium [73].

Cell culture provides an effective method for demonstrating heterogeneity, since individual cells can be grown into clones, which permits repeated testing on pure lines of cells. However, heterogeneity of cellular morphology was detected in tumors long before culture methods were available. Tumor cell heterogeneity would not be remarkable if the normal tissue were not relatively homogeneous in appearance. However, normal cells explanted in culture exhibit just as broad a range of heterogeneity as do tumor cells. This cannot be taken to mean that heterogeneity is as great in the intact tissue. As already noted, chick embryo mesenchymal cells are relatively homogeneous in size when first dispersed as single cells, but they become much larger and very heterogeneous in size if kept for 24 hours in a growth-promoting medium [4]. The distribution of albumin among cells in the intact rat liver is uniform [21], but it becomes heterogeneous within 24 hours of culturing the dispersed cells [18]. It seems apparent, therefore, that cells organized into a tissue are under the hierarchical control of the tissue and the organism that regulates their function and composition. When they are removed from their organismal context, they exhibit their intrinsic capacity for variation. Tumor cells, on the other hand, exhibit heterogeneity of structure and composition when in the organism. It is of considerable interest that the removal and dispersal of mammary epithelium from C3H mice, followed immediately by the inoculation of the dispersed cells into the fat pad, accelerates the onset of spontaneous neoplastic growth so that it can be detected in 2 months rather than the usual 9 months [82]. One implication of these observations is that naturally occurring tumors may result from the diminution of the hierarchical organizing capacity of the tissue and organism, particularly in the most common cancers, which are strongly associated with the aging process. With a decrease in effectiveness of the organizing field, the individual cells would be freer to express their innate capacity for variation. The cells might then express, in a limited, gradual sense, what they do so strikingly when dispersed for cell culture. As the cells became increasingly abnormal, the organizing effect of the field would be further compromised, and a positive feedback might result in progression of the disorganized state. This hypothetical sequence would finally express itself in a variety of changes leading to malignant behavior, including genetic and epigenetic changes in prospective tumor cells, but the driving force would have been the loss of hierarchical control. As Paul Weiss [2] has so aptly pointed out, an outstanding characteristic of living systems is the dominance of macrodeterminism. That is, the fate of large groups of cells in development and function is more highly deterministic than that of the individual cells, and much more so than biochemical processes within the cell. Contrary to the common view, it is the organism that determines, or at least limits, what DNA does, rather than the other way around. In this view, DNA serves as keys for the piano, but the organism calls the tune.

The most important concept that arises from the realization of the enormous capacity cells have for variation has to do with how they respond to change in their microenvironment. Elsasser was the first to point out the immense capacity for variation in cells based on a consideration of the number and kinds of atoms per cell, and the number of chemical bonding combinations that are possible [1, 13]. Since this number is many orders of magnitude larger than the number of elementary particles in the universe, he concluded that no two cells in the history of life on earth have ever been identical in a rigorous sense. The composition and

behavior of living cells can represent only an immensely small fraction of the combinations theoretically possible. Those actually found exist through a process he terms creative selection. Because this process involves selection from such a large number of possibilities, and because of the 'unfathomable' complexity of the processes involved, he concludes that causal chains cannot be traced beyond a terminal point. Our brief consideration of the extent of cellular heterogeneity supports Elsasser's estimate of the immensity of cellular heterogeneity. We will now consider how change with varying degrees of stability is brought about.

A broad spectrum of variable properties exists in every cell population, including surface antigens, resistance to drugs, and ability to multiply in low concentrations of growth factors, and other properties that have yet to be explored. Under constant environmental conditions, individual cells undergo transient changes in these properties, the extent of which is determined by the particular condition [57]. When the environment is altered by introducing certain constraints such as antiserum to surface antigen, low concentrations of drugs, or reduced concentrations of nutrients or growth factors, there is a selection of those spontaneously occurring transient states that are suited to function under the particular constraint. Successive steps of improved functioning can be selected in this way. At the same time the other cells, initially in a suboptimally functioning state, also continue to vary unless they slip below the metabolic threshold required for change. By this process, more and more cells are drawn into states better suited for functioning in the new environment. If a cell goes through a progression of such changes in state, the probability of return to its original state if the non-selective conditions are restored is progressively reduced. Given that each change represents selection from an immense number of possibilities, the likelihood of retracing the same steps is remote. However, the same pathway does not have to be retraced exactly to improve function. Other pathways lead to the same phenotypic effect. This would account for the observations that the Dauermodifikationen or long-lasting changes induced in Paramecium may be effectively reversed [24, 25], and that transformed mammalian cells can revert to phenotypically normal behavior [34, 73, 77, 78, 83]. The process of forward and backward selection of cellular states was first inferred from work in bacteria, where it was termed 'selection of reaction patterns' [84–86]. With the discovery of heterogeneity as a fundamental characteristic of the living state by Elsasser, and the immense number of molecular combinations that can be generated in cells, the term 'progressive state selection' was introduced to provide a sense of the dynamic flux that underlies change. I suggest that progressive state selection plays a dominant role in the progressive epigenetic changes of normal development and in the origin of cancer, with the selective conditions determined at transcellular levels. The concept of progressive state selection in no way rules out genetic changes as part of the progressive state selection in tumorigenesis. Nor does it rule out the disruptive effect of carcinogens on cell responsiveness, although it does not require the disruption to be mutational. Thus, x-rays [87–89] and chemical carcinogens [90] have been shown to heritably change the behavior of the majority of surviving cells in culture, indicating that mutations, which presumably occur at a much lower rate, are not the dominant effect of these treatments.

It should be noted that the changes associated with heterogeneity are quantitative, unlike the essentially qualitative changes associated with mutation. For example, a population of hamster cells made phenotypically resistant to bromodeoxyuridine consists of cells with a 10^5-fold range of growth capacities in selective medium [53]. The state selective process does not give rise to a uniform population. A population of NIH 3T3 cells that has adapted to low serum concentrations consists of cells with a wide range of capacities for transformation [34]. In other words, the population remains as heterogeneous as it was before the selective conditions were imposed, but the variation is at a higher level of adaptation. The concept of progressive state selection emphasizes the interaction between the cell and its environment, and is therefore properly considered holistic. But because it is based on the quantitative relations between cells and th ir

surroundings, it qualifies as a scientific rather than a philosophical or poetic holism; it falls within the generally accepted view that all truly scientific propositions are purely relational [91]. And because it is purely formal (i.e., pertaining only to arithmetic and abstract logic), it is independent of a philosophical or non-scientific background, and free of any sensory experience [1]. It therefore has the potential for broad generalization in biology, much as the formal propositions of physics do in the physical sciences.

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#15

Review–Hypothesis

Primary culture, cellular stress and differentiated function

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Isolation of specialized cell types for the analysis of tissue-specific gene function often results in loss of the differentiated phenotype. Examples of this type of phenotypic change following tissue disaggregation are reviewed together with possible explanations. Close similarities between the effects of cell isolation with those of other cellular stresses such as heat or anoxia point to common biochemical mechanisms being involved. This suggests that the study of freshly isolated cells will contribute significantly to out understanding of the nature of cellular stress and its consequences for the maintenance of phenotype and induction of tissue specific gene expression.

Tissue disaggregation *Cellular phenotype* *Hormonal responsiveness* *Heat shock protein* *Culture shock*

1. INTRODUCTION

Primary culture of differentiated cells is now widely used to study the phenotypic expression of specialized tissue-specific functions. These studies are often vitiated by the loss of differentiated characteristics, either very shortly after tissue disaggregation and cell isolation or during the subsequent maintenance of the cells in vitro over periods of days and weeks. Although the loss of phenotype during prolonged maintenance of cells in culture has been extensively observed [1,2], little emphasis has been placed on the more immediate consequences of cell isolation. This review concerns the transient loss of specialized function usually encountered in freshly isolated cells for primary culture and emphasizes the strong similarities with the effects on cellular phenotype following induction and recovery from the well known response to heat shock and other cellular stresses [3].

2. CELL ISOLATION

As the loss of cellular phenotype often occurs during or immediately following cell isolation, it is essential to outline the procedures involved. Primary cell cultures are commonly prepared either by allowing cells to migrate out of fragments of tissue adhering to a suitable substrate or by disaggregating the tissue mechanically or enzymatically to produce a suspension of cells, some of which will ultimately attach to the substrate. In general, the requirement for high yields of cells means that enzymatic digestion of the tissue biomatrix is the method of choice [4,5]. Crude trypsin is the enzyme most frequently used for embryonic tissue, but is widely recognized to damage membranes and alter normal cellular function [6,7]. The large amounts of fibrous connective tissue in adult organs has led to the use of collagenase in isolating terminally differentiated cells [4,8]. The concentration of enzyme, the method of tissue dispersal and the centrifugal force used for washing the cells all greatly influence the final yield of viable cells.

Liver parenchymal cells have been more extensively studied in primary cell culture than any other cell type. These cells are particularly susceptible to trauma, their isolation requiring the use of the

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gentlest methods available [5,9]. Berry and Friend [10] established the basic protocol involving a two-step perfusion of the liver in situ, first with calcium-free medium, followed by a calcium-rich medium containing collagenase. Tissue disaggregation is then continued in vitro most commonly by finely chopping up the tissue and agitation in enzyme-containing medium.

In earlier studies, the perfusate was intensively oxygenated but Seglen [11] omitted oxygenation to facilitate sterility without any apparent deleterious effects [12]. The use of high concentrations of Hepes (20 mM) to buffer the perfusate maintains the yield and quality of cells, as judged by morphology, respiration and several hormonally responsive functions [13,14]. Cells isolated in the absence of supplementary oxygen show some discrete morphological alterations, such as retraction of the mitochondrial matrix and abnormal appearance of endoplasmic reticulum, similar to those seen in rat liver parenchymal following hypoxia in vivo, although these features are readily reversible [15].

Tissue disaggregation by the collagenase digestion procedure has also been used for preparation of cells from other epithelial [16–18], mesenchymal [19,20] and endocrine tissues [21]. The tissue-specific functions of these cells are then either analysed immediately or more commonly during the first few days in culture.

3. LOSS OF PHENOTYPE OF CELLS IN SUSPENSION OR SHORT TERM CULTURE

Investigation of cellular function during the first 24 h after isolation often involves the maintenance of isolated cells in suspension, during which time they are agitated to provide both oxygen and prevent aggregation [22,23]. Alternatively, they may be allowed to settle onto a substratum to which they attach rapidly and, in the case of liver parenchymal cells, reform tissue characteristic structures such as trabeculae and bile canaliculi by the second day in culture [24,26].

The viability of isolated cells has been assessed in various ways: vital dye exclusion, lactate dehydrogenase leakage [27], fine structural analysis [14], attachment to the substratum [28], respiration [11], gluconeogenesis [14] and the capacity to respond to various hormonal and other stimuli (see below). Several laboratories ha adopted the maintenance of normal levels cytochrome P450 as a criterion for liver pare chymal cell viability [29,30]. Rat hepatocyt cultured for 24 h lose 70% of their cytochron P450 [31,32], while in bovine adrenal zo glomerulosa cells the loss of capacity to synthesi aldosterone accompanies the decline in cyt chrome P450 following isolation [33,34]. Th decrease can be prevented by supplementing t culture medium with a variety of nutrients, grow factors and hormones [35,36], or by the incorpor tion of hyperphysiological concentrations nicotinamide or pyridine derivatives (metapyron in the culture medium [37,38].

Almost all mouse liver-specific mRNAs declir following parenchymal cell isolation due to inhib tion of their transcription [39]. Of all hepat mRNAs the best studied gene product in prima cell cultures is serum albumin [26,40,41]. Fifty pe cent of total plasma protein synthesis in liver devoted to albumin both immediately after ce isolation and in vivo [42–44]; its synthesis an secretion rapidly decline to negligible levels durin the first 2–3 days in culture [45,46]. This reductio in synthesis is due to both a rapid decline in cellul albumin mRNA levels [26] and perhaps transl tional control as well [47]. Addition of insulin t the culture medium partially alleviates this declir in both mRNA levels and albumin synthes [46–48], while serum itself appears ineffecti [45]. The relatively enhanced synthesis of oth adult type plasma proteins during maintenance c cells in culture (see below) indicates that the declir in albumin synthesis is selective and not due to general loss of cellular viability as seen in r hepatocyte suspensions [22,49].

Although the synthesis of cytochrome P450 an albumin represents the best documented examp of loss of constitutively synthesized tissue specif proteins in short term culture, other examples a also known, including both secreted and i tracellular proteins [50,51], where specific protei synthesis rapidly decreases following cell isolatio

It should be emphasized that the above losses o cur in the first 2–3 days of cell culture and the short term studies should be distinguished fro long term cultures. In the latter, phenotyp changes may be associated with selection of mo actively proliferating cells under the limitations

nutrient, hormone and substrate availability [1,8].

The isolation procedure removes the cell type of interest from its normal environment leading to the loss of identifiable plasma membrane domains [52]. Epithelial cells are found to be rounded and dispersed singly or in small clumps [24,26]. Maintenance of normal in vivo levels of constitutively synthesized tissue specific proteins is significantly enhanced by culturing these cells on or within specific biomatrices such as collagen gels [53,54], or matrices from other natural tissue glycoproteins such as fibronectin [50]. Complex biomatrices have also been employed, as for example, that secreted by bovine corneal epithelial cells [41], or isolated from rat liver [55]. However, liver parenchymal cells are able to synthesize their own collagen biomatrix in culture [56]. Albumin synthesis can be sustained in long term cultures (>20 days) by co-culturing liver parenchymal cells with another liver epithelial cell type [57,58], however in these studies it is clear that the rate of albumin synthesis increases during maintenance in culture to peak values at 10 days in the presence of fetal calf serum which may reflect hormonal induction, and also a partial recovery of rates of synthesis and secretion to those found in vivo.

4. HORMONAL RESPONSIVENESS IN SHORT TERM CULTURES

Perhaps the best indicator of the maintenance of differentiated function after isolation of cells is their ability to respond to specific hormones that rapidly modulate metabolic activity or promote their growth and development [59]. It is the rapid and transient metabolic actions of hormones such as glucagon and insulin which have been widely studied in isolated cells. However, hormonal responsiveness is often reduced or even lost as a consequence of a reduction [60], or even elimination [61], of the number of hormone receptors on the cell surface immediately following cell isolation. Following establishment of primary cell cultures hormone receptor numbers gradually increase, leading to the recovery of full hormonal responsiveness [60,61]. This recovery appears to be dependent on RNA and protein synthesis. Proteolysis alone appears inadequate to explain these decreases in cell surface receptors, since isolation of cell membranes without the use of collagenase [62] also results in receptor loss. Hormone binding is also known to decline following surgical trauma such as partial hepatectomy [63].

The effects of addition to cultured cells of hormones that regulate metabolic activity (i.e., insulin and glucagon) are usually rapid and often act via changes in cAMP metabolism [64] or metabolite transport processes at the plasma membrane [65,66]. Rapid and transient metabolic actions of hormones acting at the plasma membrane would appear therefore to be retained or rapidly recovered in cell suspension or primary cell culture, maximal hormonal responsiveness being achieved 48 h after cell isolation [67].

The actions of hormones with relatively slow growth and developmental actions has been more difficult to study in isolated cells, and hence less extensively investigated. One problem is the often rapid metabolism of the hormone in the target cell. For example, triiodothyronine [68] and steroid hormones [69,70] are rapidly metabolized in hepatocytes, thus accounting for the requirement for high doses of hormone in eliciting a full physiological response [24,71]. Frequent replenishment of the culture medium with the hormone may entirely alleviate variable or suboptimal responses [69,71] allowing the same quantitative hormonal effects as seen in vivo [72,73].

As is the case for immediate metabolic effects, hormonal effects on protein and RNA synthesis are also reduced following cell isolation, even if adequate hormone concentrations are maintained in primary culture [26,74–79]. In general, response to growth and developmental hormones recovers to maximum levels after 5–10 h in suspension culture [22,75,76] or after 2–3 days in primary cell culture on fixed substratum [24,26,45].

The levels of hormonally regulated tissue specific mRNAs and proteins may rapidly decline following cell isolation, e.g., albumin [46] or α_{2u}-globulin [80]. Glucocorticoids, which are known to enhance albumin synthesis [81,82], help to maintain its rate when added to hepatocytes soon after their isolation [50,83]. In this respect the full induction of vitellogenin synthesis in primary cultures of amphibian or avian liver parenchymal cells offers many advantages, such as the competence of male liver to respond to estrogen, offering a 'zero' background of prior vitellogenin gene expression and being independent of cell division

or DNA synthesis [72,84,85]. The levels of certain fetal isozymes and α-fetoprotein are known to increase following cell isolation, a process often termed 'dedifferentiation' or 'retrodifferentiation' [86]. The acquisition of a fetal phenotype [8] is, however, an unlikely explanation for loss of specialized, adult cell functions. These fetal characteristics are associated with rapidly proliferating hepatocytes and the rate of DNA synthesis may determine their expression [87,88]. It is also important for this discussion to realise that 'dedifferentiative' changes in phenotype of this kind to not occur until 3–6 days after cell isolation.

There are many similarities between hormonal induction of gene expression and the induction by drugs of detoxicating enzyme systems [89,90]. For example, phenobarbitone is ineffective in increasing cytochrome P450 levels for the first 24 h after isolation of liver parenchymal cells from normal or hepatectomized adult rats [30,31]. The capacity of the cells to accumulate cytochrome P450 in response to phenobarbitone has been shown to increase to a maximum level only after 4 days of culture [91].

In addition to its rapid metabolic effects on isolated cells, insulin enhances the relatively slow lipogenesis in short term rat hepatocyte primary cultures [92,93], the action requiring continuing RNA synthesis following a 24-h lag period after cell isolation [92,94]. A similar latent period was observed with the stimulation of lipogenesis by triiodothyronine in primary cultures of chick embryo hepatocytes [25].

In summary, following cell isolation there is quite commonly an extended lag period during which the response of cell cultures to growth and developmental hormones is slow and suboptimal in comparison to the in vivo response. The specialized tissue-specific functions generally recover, albeit not fully in all cases, to give maximal hormonal responsiveness after usually 2–3 days in culture.

5. CULTURE SHOCK AND THE LOSS OF DIFFERENTIATED FUNCTION

Explanations offered for the loss of specialized tissue specific gene function following cell isolation and short term primary culture include: (i) removal of tissue substratum [95]; (ii) nutrient and hormonal deprivation [2]; (iii) onset of DNA synthesis or cell division [87]. These suggestions however, do not provide a biochemical mechanism. Furthermore, some of the above factors are not associated with the loss of phenotypic function, e.g., in *Xenopus* liver parenchymal cells which do not divide in culture [26] and the failure of cell–cell contact, cell substratum interaction, and hormone or growth factor supplemented serum to prevent short term loss of phenotype in mouse hepatocytes [39].

An important factor influencing protein and nucleic acid metabolism is cell morphology [96,97]. Reattachment of mouse fibroblasts in suspension culture to a substratum results in a rapid recovery of overall protein synthesis within a few hours, although complete restoration of mRNA, rRNA and DNA synthesis require about 18 h of culture after reattachment. This recovery process is dependent on extensive cell spreading and appears to be shape dependent. In these fibroblasts, actin mRNA levels are specifically down-regulated following detachment from the substratum. Similar recoveries in protein and nucleic acid metabolism are seen following attachment of cells isolated for primary culture to the tissue culture dish surface [98,99].

Another important factor, overlooked until now, and relevant to cell structure, is the increase in stress protein synthesis in freshly isolated cells recently characterized in our laboratory [26]. The synthesis of stress or heat shock-like protein declines during the first 2–3 days in culture concomitantly with the reformation of cell–cell contacts and substratum interactions. These proteins are known to associate with cytoskeletal elements [100–102] leading to alterations in cell morphology [26,103] that are reversible on return to the normal incubation temperature.

Stress proteins may be synthesized in embryonic tissue at normal temperatures and are generally thought to be constitutively synthesized at low levels in unstressed tissues [104–106]. Although the only established role of heat shock proteins is in thermotolerance [107], diverse forms of stress lead to the induction of their synthesis. Many of these stresses are produced during cell isolation: (i) mechanical trauma [108,109]; (ii) deprivation of glucose [110,111]; (iii) potential influx of Ca^{2+}

[112]; (iv) anoxia [113]. Anoxia (and recovery from it) is particularly relevant to the isolation of cells, since many tissues used for primary cell culture have a high requirement for oxygen, in particular liver. Highly differentiated cell types might be deprived of oxygen both during preparation or subsequent maintenance in culture [114]. Stevens [115] proposed that growth in primary cell culture was limited due to the high requirement for oxidative metabolism in isolated hepatocytes in comparison to most secondary cell lines or fibroblasts.

Most highly differentiated cells stop synthesizing normal or non-heat shock mRNAs or proteins upon trauma or stress [116–120]. Pre-existing mRNAs are either sequestered in a non-translatable form or degraded, while recovery of normal protein synthetic patterns usually requires 12–36 h after return to normal temperature [119,120]. There may also be a transient paralysis in hormonal responsiveness during this period [120].

There are therefore many similarities between the effects of heat shock and cell isolation, which may point to common biochemical mechanisms. For example, isolation of rat liver parenchymal cells results in estrogen receptor levels falling to 50% of the in vivo level, with a further 17% fall in the first hour of suspension culture [51]. In *Xenopus* liver parenchymal cells, heat shock leads to the disappearance of estrogen receptor, its synthesis explaining the 24–36-h lag period of estrogen responsiveness following return to normal temperature. Addition of estrogen prior to heat shock prevents this loss, therefore allowing an immediate hormonal response [120]. Similar protection by ligand is seen with the stabilization of cytochrome P450 by pyridine derivatives following hepatocyte isolation [38]. Heat shock leads to the dephosphorylation of ribosomal protein S6 [121,122], which is the same protein whose dephosphorylation following establishment of primary cultures selectively affects albumin synthesis [47].

If the inhibition of tissue-specific function following both cell isolation and heat shock are due to common mechanisms following cellular stress then monitoring cellular protein synthetic patterns will indicate the degree of stess. It may be presumed that maximal hormonal response or recovery of specialized function will not occur until the cells have recovered from the stress which would be indicated by the decline in stress protein synthesis. The study of heat shock has concentrated on the mechanisms of induction of stress proteins, whereas cell isolation studies have focussed on the maintenance of normal differentiated function. If common biochemical mechanisms are involved in both then the study of isolated cells will contribute substantially to our understanding of cellular stress and its effects on normal differentiated function. At the same time, the recognition of cellular stress will allow the full exploitation of primary cell cultures in studying the regulation of phenotypic function.

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